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Mechanisms of In Vitro Sensitivity to Sulfadiazine Silver

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• **Sulfonamide-resistant organisms have been reported as a frequent consequence of the clinical use of sulfadiazine silver. At this burn center, sulfonamide resistance occurred in more than 80% of gram-negative isolates. We tested the requirement for the individual antimicrobial activities of sulfadiazine and silver for in vitro activity of sulfadiazine silver. The sulfadiazine component is not necessary for in vitro sensitivity. In vitro sensitivity to sulfadiazine silver does not consistently predict the presence of therapeutic activity in *Pseudomonas aeruginosa*–infected rats with burns. We describe an example of a transferable multiple-antibiotic resistance plasmid that contains selectable sulfonamide resistance. The use of sulfadiazine silver can, therefore, lead to the selection of organisms that are resistant not only to sulfonamides but to antibiotics of clinical consequence, and this possible risk must be considered in electing to use the agent.**
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The silver salt of sulfadiazine is the chemotherapeutic agent most commonly used in the topical treatment of hospitalized patients with burns. Shortly after first using this agent, Lowbury et al[1] observed increased sulfonamide resistance among gram-negative organisms isolated from burns. We have noted a similar phenomenon. At our burn center, sulfadiazine (in sulfadiazine silver salt) is the only bacteriologically active para-aminobenzoic acid antagonist in clinical use.

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Despite several reports of in vitro assays of the antimicrobial activity of sulfadiazine silver, the specific role of sulfadiazine in the antibacterial activity of this salt has not been defined.[2-4] The question of whether sulfadiazine plays a direct antibacterial role as a sulfonamide, behaves as a chemical coordinator of silver ion, or has both functions in vitro has not been answered. We have used a bacteriologically inactive analogue of sulfadiazine, 2-benzenesulfonamidopyrimidine (ISR-44), as a control molecule to test the specific silver and sulfonamide activities of sulfadiazine silver in vitro.[5] The analogue ISR-44 has been shown to have silver-coordinating properties similar to sulfadiazine.[6] Sulfadiazine and ISR-44 were examined in vitro as both sodium and silver salts and also in vivo, using rats with burns that were infected with sulfonamide-sensitive and a sulfonamide-resistant strain of *Pseudomonas aeruginosa*.

We report our findings, define the sulfonamide requirement for in vitro activity of sulfadiazine silver, and address the significance of in vitro sensitivity to sulfadiazine silver in therapeutic activity against experimental *P aeruginosa* burn wound sepsis. Additionally, we present an example of the potential risk inherent in maintaining sulfonamide resistant plasmids in the clinical burn environment.

MATERIALS AND METHODS

The analogue ISR-44 was synthesized by condensation in pyridine of benzene sulfonyl chloride and 2-aminopyrimidine.[7] The precipitated ISR-44 was filtered, washed repeatedly with water, and lyophilized. The sodium salt of ISR-44 was prepared by solubilizing free-base ISR-44 with aqueous sodium hydroxide and lyophilization. Sulfadiazine sodium was purchased from a commercial manufacturer. Silver salts of ISR-44 and sulfadiazine were prepared by mixing equal molar concentrations of aqueous solutions of the sodium salts and silver nitrate.[8] The precipitated silver

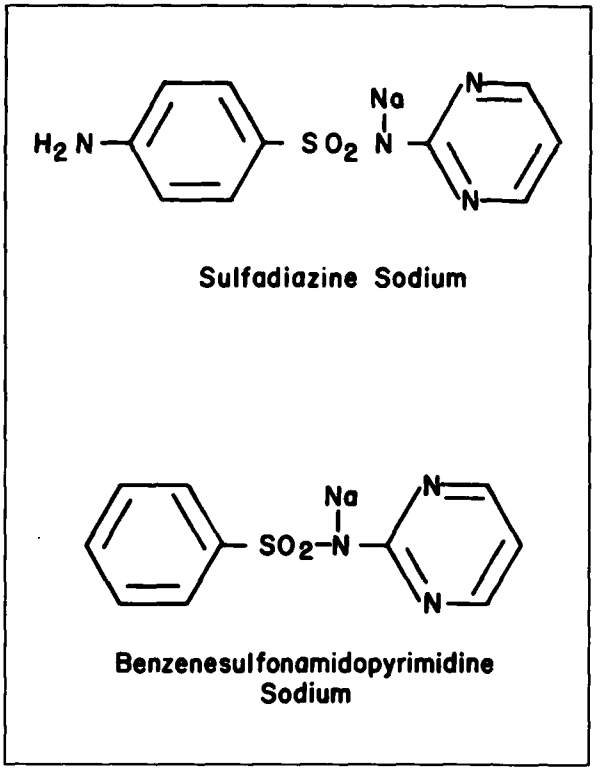

Fig 1.—Structures of sulfadiazine sodium and benzenesulfonamidopyrimidine sodium.

salts were repeatedly washed with water and lyophilized. The silver salt of ISR-44 was designated ISR-45.

In vitro sensitivity assays were performed by trench plate or agar well techniques. Trench plates were prepared by cutting trenches (width, 0.5 cm) in 15-cm Mueller-Hinton agar plates (using parallel scalpel blades). The agar pieces were removed, the trenches were filled with test compound in 1.5% molten agar (50 °C), and the plate was then overlaid with 20 mL of Mueller-Hinton agar. After solidification, test plates were cross-streaked with clinical isolates of sulfonamide-sensitive or -resistant gram-negative bacteria. Sulfonamide sensitivity was also determined by disc diffusion technique.[8] Agar well plates were prepared by punching 1-cm wells into 15-cm Mueller-Hinton plates.[9] After removal of the agar plug, the well was filled with a test compound in 1.5% molten agar, and after solidification, the plates were overlaid with 9 mL of 1.5% molten agar that contained a test organism at 10^5 viable organisms per milliliter. All in vitro testing was performed with test compounds at 50 mg/mL.

The relationship of in vitro activity of sulfadiazine silver to in vivo chemotherapeutic activity was investigated in *P aeruginosa*–infected rats with burns.[10,11] The Walker-Mason scald model was used with 180 to 200-g Sprague-Dawley rats. Full-thickness injuries of 20% of the total body surface were inflicted by exposure of the dorsum of anesthetized rats to boiling water for 10 s. After scalding, animals were inoculated with 1 mL of medium containing 10^6 viable organisms. Two *Pseudomonas* strains were investigated. Strain 59-1244 is sensitive in vitro to sulfonamides, and its rat virulence has been previously described.[11,12] The second strain, 70-4189, is a burn patient isolate that is virulent in the rat model and resistant in vitro to sulfonamides. Topical chemotherapy was started 24 hours after burning and inoculation and continued once per day for ten days. Mortality was recorded for 28 days after burning. All test compounds were suspended in an ointment base.

Providencia stuartii has reappeared at our institute as a serious opportunistic burn pathogen after several years' absence.[13] The index case was a patient with burn injuries who was transferred from a South American burn treatment facility; at admission, an isolate identified as *P stuartii* was yielded from the patient's blood culture and found to be resistant in vitro to aminoglycosides, tetracyclines, chloramphenicol, ampicillin, carbenicillin, polymyxin B, mercuric chloride, and sulfonamides. This strain was tested for the ability to transfer multiple-drug resistance associated with sulfonamide resistance to *Escherichia coli* K12 strain C600. Strain C600 is free of the *E coli* sex factor F and phage lambda. The strain is genetically identifiable, and an additional nalidixic acid chromosomal resistance mutation was added at our institute. The nalidixic acid–resistant strain was designated C601. The *Providencia* isolate was found to be sensitive to nalidixic acid.

Transfer of antibiotic resistance was effected by a filter-mating technique.[14] After incubation of the filter that supported a mixture of the *Providencia* isolate and C601 on nutrient agar for six hours, the filter was vortexed in broth, diluted, and plated on double-selective media that contained nalidixic acid and an antibiotic to which the *Providencia* isolate was resistant and to which C601 was sensitive. All antibiotics, except sulfadiazine, were prepared in tryptic soy agar plates. Sulfadiazine-selective plates were made using Mueller-Hinton agar. Control plates contained single antibiotics that were selective for one of the mating types. These plates allowed quantitation of the donor and C601 in the mating. Frequency of transfer was calculated as the ratio of transconjugates to donors in the original mating mixture.

Parent and transconjugate strains were examined for extrachromosomal DNA elements by 0.7% agarose electrophoresis of partially purified DNA cell lysates, and DNA extracts were prepared by a modification of the technique of Meyers et al.[15] Protease (type 14) was used in place of lysozyme, and phenol extraction was eliminated.

RESULTS

The structures of sulfadiazine sodium and ISR-44 sodium are presented in Fig 1. As can be seen, the two compounds have similar structures, except that ISR-44 does not contain an N,4 amino group. This deletion renders the molecule inactive as a *p*-aminobenzoic acid competitor and, therefore, bacteriologically inactive as a sulfonamide. The in vitro activities of sulfadiazine sodium, ISR-44 sodium, sulfadiazine silver, and ISR-45 (ISR-44 silver) against sulfonamide-sensitive gram-negative burn isolates are shown in Fig 2. The bottom two streaks on each plate are control sulfonamide-sensitive cultures. As predicted, ISR-44 has no demonstrable antibacterial activity. All strains were inhibited by the sulfadiazine-containing trench. The silver salts of both compounds produced inhibition. The sulfadiazine silver trench exhibited a wider zone of inhibition than the ISR-45 trench. The in vitro activity of the four test compounds against sulfonamide-resistant gram-negative isolates is shown in Fig 3. The bottom two streaks again are control-sensitive strains. Isolates resistant to sulfonamides by trench plate assay were also resistant to sulfadiazine sodium by disc diffusion. Silver salts of both compounds were active against all strains. The zone of inhibition above the sulfadiazine silver trench was similar to

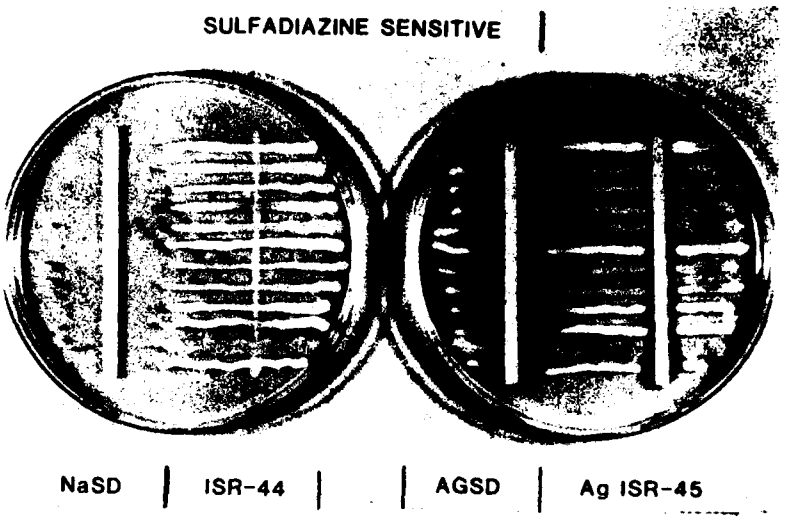

Fig 2.—Trench plate assays using sulfadiazine (sulfonamide)–sensitive gram-negative isolates from patients with burn injuries. Trenches contain test compounds at 50 mg/mL. NaSD indicates sulfadiazine sodium; ISR-44, 2-benzenesulfonamidopyrimidine sodium; AGSD, sulfadiazine silver; and AgISR-45, 2-benzenesulfonamidopyrimidine silver.

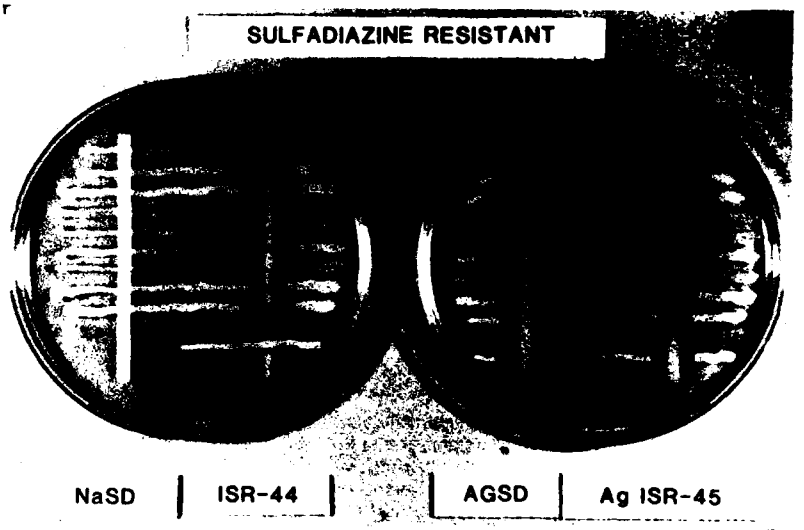

Fig 3.—Trench plate assays using sulfadiazine (sulfonamide)–resistant gram-negative isolates from patients with burn injuries. Trenches contain test compounds at 50 mg/mL. Bottom two streaks on both plates are control sulfonamide-sensitive cultures. For explanation of abbreviations, see legend for Fig 2.

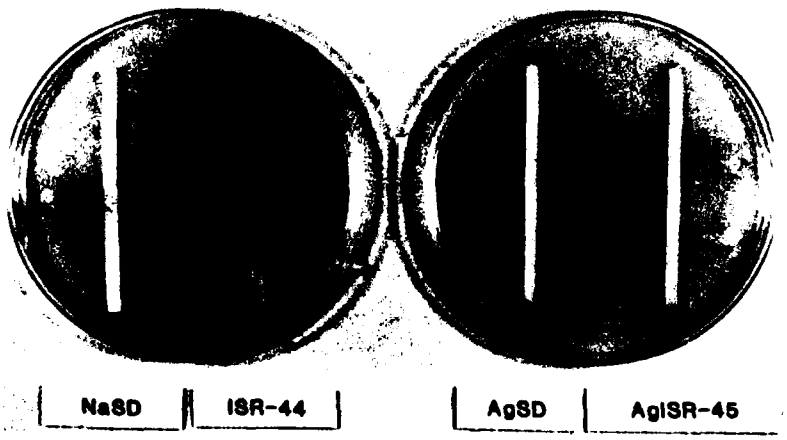

Fig 4.—Trench plate assay of burned rat virulent *Pseudomonas aeruginosa*. Top streak on both plates is strain 59-1244. Bottom streaks are strain 70-4189. Trenches contain test compounds at 50 mg/mL. For explanation of abbreviations, see legend for Fig 2.

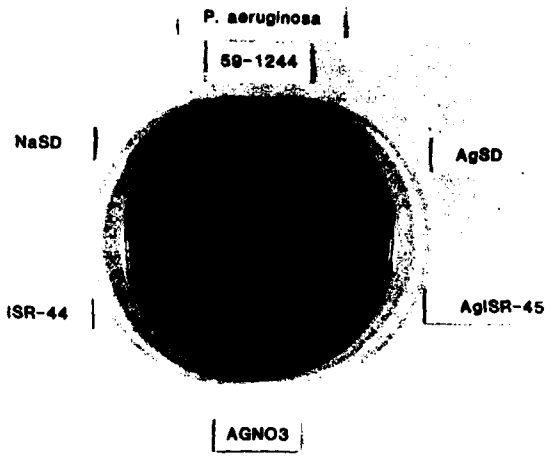

Fig 5.—Agar well assay of strain 59-1244. Silver nitrate ($AgNO_3$) is in center well. Wells contain test compounds at 50 mg/mL. For explanation of abbreviations, see legend for Fig 2.

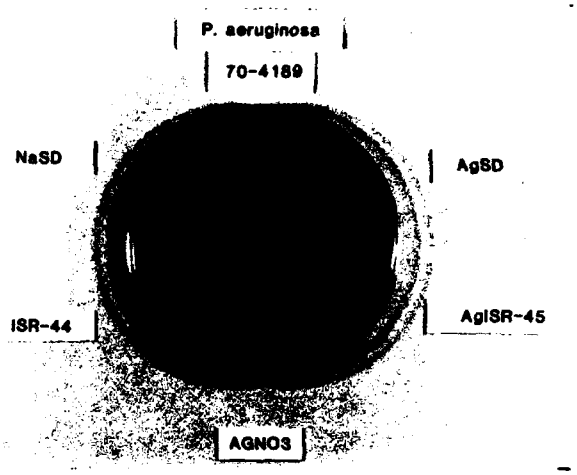

Fig 6.—Agar well assay of strain 70-4189. Silver nitrate ($AgNO_3$) is in center well. Wells contain test compounds at 50 mg/mL. For explanation of abbreviations, see legend for Fig 2.

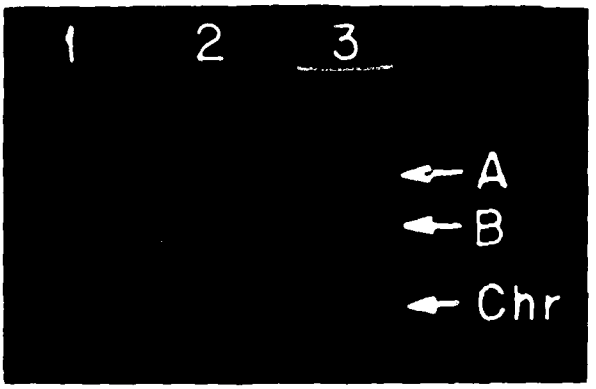

Fig 7.—Agarose gel electrophoresis of partially purified DNA from *Escherichia coli* C601 (track 1), *Providencia stuartii* (track 2), and transconjugate strain resulting after filter mating and selection for sulfonamide resistance transfer (track 3). Band A is plasmid DNA with molecular weight of approximately 80 megadaltons; band B, plasmid DNA with molecular weight of approximately 35 megadaltons; and band Chr, fragmented chromosomal DNA.

Table 1.—Topical Chemotherapy in Experimental *Pseudomonas* Burn Infection of Rat

Treatment*	Mortality, %	
	Strain 59-1244	Strain 70-4189
Sulfadiazine sodium, 10 mg/g	0	90
Sulfadiazine sodium, 50 mg/g	0	50
ISR-44 (sodium), 50 mg/g	100	100
Sulfadiazine silver, 50 mg/g	0	80
ISR-45 (silver), 50 mg/g	100	100
Mafenide acetate, 112.5 mg/g	10	10
Infected (no other treatment)	100	100

*Treatment was started 24 hours after burning and inoculation and continued once per day for ten days. Ten animals were used per group, and mortality was recorded for 28 days.

the zone above the ISR-45 trench. This is perhaps the result of loss of the effect of the sulfonamide component of the salt.

Two strains of *P aeruginosa* that are virulent for rats with burns were examined for in vitro sensitivity. Data are shown in Fig 4. The top streak is strain 59-1244. This strain is sensitive to sulfonamides by disc technique and was inhibited by sulfadiazine sodium. The ISR-44 had no activity. Both silver salts inhibited strain 59-1244. The bottom streak is strain 70-4189. This strain is resistant to sulfonamides by disc technique and was not inhibited by the sulfadiazine sodium trench. Strain 70-4189 was sensitive to the silver salts. These two strains were also tested for sensitivity by the agar well technique. Figure 5 shows strain 59-1244 to be sensitive to sulfadiazine sodium, sulfadiazine silver, ISR-45, and silver nitrate (center well). The ISR-44 had no activity. Figure 6 shows strain 70-4189 in the agar well assay. This strain was sensitive to the three silver compounds but resistant to sulfadiazine sodium and ISR-44.

The in vivo correlation of in vitro testing of these strains was tested by chemotherapeutic trials of sulfadiazine sodium, ISR-44, sulfadiazine silver, and ISR-45 creams. Data are given in Table 1. Strain 59-1244 was sensitive to topical sulfadiazine sodium but resistant to ISR-44 sodium. Strain 70-4189 was resistant to treatment with sulfadiazine sodium and also resistant to ISR-44 sodium. These data correlate well with the in vitro observations. A surprising finding was that despite in vitro sensitivity to ISR-45, both *Pseudomonas* strains were resistant in vivo. Strain 70-4189 was also resistant to sulfadiazine silver, a resistance also in contrast with in vitro findings.

A single linkage of multiple antibiotic resistance was transferred from the *Providencia* isolate to *E coli* C601. Data are given in Table 2. Independent selection with chloramphenicol, gentamicin, kanamycin, and sulfadiazine yielded similar results. Selection with these drugs showed a pattern of ampicillin, carbenicillin, chloramphenicol, gen-

Table 2.—Antibiotic Resistance Markers Transferred From *Providencia stuartii* to *Escherichia coli* C601

Selective Media	Phenotype Transferred	Transfer Frequency
Chloramphenicol (25 mg/L) + nalidixic acid (100 mg/L)	Ampicillin, carbenicillin, chloramphenicol, gentamicin, kanamycin, mercuric chloride, sulfonamide-sulfadiazine, tobramycin	4.5×10^{-5}
Gentamicin (25 mg/L) + nalidixic acid (100 mg/L)	Ampicillin, carbenicillin, chloramphenicol, gentamicin, kanamycin, mercuric chloride, sulfonamide-sulfadiazine, tobramycin	1.8×10^{-5}
Kanamycin (50 mg/L) + nalidixic acid (100 mg/L)	Ampicillin, carbenicillin, chloramphenicol, gentamicin, kanamycin, mercuric chloride, sulfonamide-sulfadiazine, tobramycin	2.3×10^{-5}
Sulfonamide-sulfadiazine (50 mg/L) + nalidixic acid (100 mg/L)	Ampicillin, carbenicillin, chloramphenicol, gentamicin, kanamycin, mercuric chloride, sulfonamide-sulfadiazine, tobramycin	9.0×10^{-5}
Polymyxin B (25 mg/L) + nalidixic acid (100 mg/L)	None transferred	...
Streptomycin (20 mg/L) + nalidixic acid (100 mg/L)	None transferred	...

tamicin, tobramycin, kanamycin, mercury, and sulfonamide resistance transferred at a similar frequency. Selection with streptomycin- or polymyxin-containing plates showed no transfer. These data suggest a single plasmid transfer of genetically linked resistances.

Agarose electrophoresis of partially purified DNA from the donor *Providencia* strain, *E coli* C601, and a transconjugate are shown in Fig 7. *Escherichia coli* C601 contains no detectable plasmid DNA. The *Providencia* strain was found to contain two plasmid bands. Band B has a molecular weight of approximately 35 megadaltons, and band A is a larger plasmid at approximately 80 megadaltons. The presented transconjugate, which was selected with sulfadiazine, shows a plasmid pattern identical to the donor *Providencia*. Examination of 15 transconjugates by agarose electrophoresis showed identical plasmid patterns. With the finding of an apparent single-linkage group of antibiotic resistance transfer but two plasmids, one cannot establish a definite location for resistance genes on the plasmids. A transconjugate that was selected on gentamicin-containing media was found to have a single 80-megadalton plasmid pattern (not shown). This transconjugate had the same antibiotic resistance pattern as all examined transconjugate strains. This suggests that the larger plasmid contains the antibiotic resistance genes. The smaller plasmid may represent a high-copy number plasmid of unknown function that because of its high-copy number relative to the resistance plasmid has a high probability of cotransfer.

COMMENT

In vitro assay of antimicrobial susceptibility plays a key role in antimicrobial chemotherapy. Criteria for in vitro susceptibility are established for parenteral antibiotics by pharmacokinetic measurements of achieved levels and laboratory testing to reflect the relative antimicrobial activity of the achieved levels. Such criteria do not exist for topical chemotherapeutic agents.

In this study, we have attempted to assess the role of sulfadiazine per se as an antimicrobial agent in the in vitro activity of sulfadiazine silver. Previous reports of activity of sulfadiazine silver both in vitro and in vivo have not specifically addressed this question. It is obvious from the data presented that the sulfadiazine component is not necessary for in vitro sensitivity. It also seems to be true that the N,4 amino group is not a chemical requirement for silver activity in that ISR-45 was as active as sulfadiazine silver against sulfonamide-resistant bacteria.

The value of in vitro testing for susceptibility to sulfadiazine silver is questionable. Modak and Fox[16] have recently reported that *Pseudomonas* burn infections resistant to sulfadiazine therapy are increasing. They also report that *P aeruginosa* isolated from these cases is sensitive in vitro to sulfadiazine silver. When several of these strains were tested in burned mice and rats, the infections were resistant to sulfadiazine silver. The significance of these findings is uninterpretable without examination of the specific mechanism of resistance. It seems possible that the strains tested were, as in strain 70-4189, resistant to sulfonamides but sensitive in vitro to silver salts.

The data presented in this report demonstrate that invasive infection with *P aeruginosa* strain 59-1244, which is sensitive to sulfonamides in vitro, can be effectively treated with topical sulfadiazine sodium. The same strain at the same phase of infection was not treatable with ISR-45 despite the in vitro activity of this silver salt. Strain 70-4189 was resistant to sulfonamide treatment both in vitro and in burned rats. In vitro sensitivity of this organism to both sulfadiazine silver and ISR-45 was not predictive of therapeutic activity in the rat model. If one accepts that ISR-44 is chemically similar to sulfadiazine with respect to silver coordination, these data support the concept that silver is not a therapeutically active agent in this model of *P aeruginosa* subsurface infections. We have not addressed the effects of silver salts on surface colonization by organisms, but it seems reasonable to expect that with surface colonization by organisms resistant to sulfonamide but sensitive to silver ions, both ISR-45 and sulfadiazine silver would be effective. Under circumstances where infection has occurred and the organism is resistant to sulfonamides, however, failure of penetration by active silver may limit effectiveness, as has been demonstrated for silver nitrate therapy.[17]

The intent of our study is not to question the clinical use of sulfadiazine silver. Rather, it is intended to present a clearer picture of the use of in vitro testing for sensitivity to this agent. The sulfadiazine component of the compound functions independently as an active sulfonamide, and it is recommended that sulfonamide sensitivity testing be rou-

tinely conducted when using sulfadiazine silver.

Our data indicate that selection of sulfonamide-resistant organisms by the use of sulfadiazine silver incurs a risk of concomitant selection of organisms resistant to a spectrum of clinically useful antibiotics. This risk must be considered in assessing the epidemiologic consequences of choosing to use this agent.

In conducting the research described in this report, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals*, as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

Avery A. Johnson synthesized the ISR-44 and ISR-45 used in this study.

The ointment base used in this study was supplied by Marion Laboratories, Kansas City, Mo.

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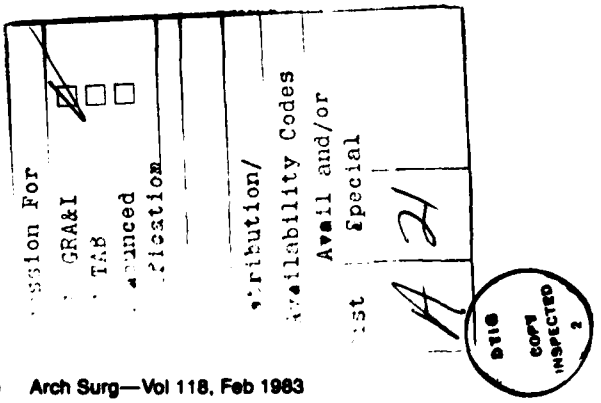
Discussion

H. STEPHEN BJORNSON, MD, Cincinnati: There has been considerable discussion in the literature regarding the relative contribution of the silver ion and sulfadiazine moieties of sulfadiazine silver to the antibacterial activity of this compound. In vitro studies have demonstrated that the silver ion interacts with the chromosomal DNA and cell membrane of bacteria. It has been suggested that inhibition of bacterial growth in vitro by sulfadiazine silver is the result of interference of DNA function by binding of silver ions along the DNA molecule; the contribution of the sulfadiazine moiety to the antibacterial activity of the compound is unclear. Minimal information is available regarding the correlation between in vitro and in vivo sensitivity of bacteria to the antibacterial effects of sulfadiazine silver. The results presented by Dr McManus and colleagues suggest that in vivo the sulfadiazine moiety may play a prominent role in the antibacterial effects of sulfadiazine silver.

I have two questions for Dr McManus. First, based on the results of the investigation, does he think that it would be advisable to determine the sensitivity of burn wound isolates to both sulfadiazine and sulfadiazine silver? Second, does Dr McManus have any preliminary data on the correlation between the in vitro sensitivity of bacterial isolates from patients with burn injuries at his institution to sulfadiazine, sulfadiazine silver, and the effect of topical therapy with sulfadiazine silver on the flora of the burn wound?

DR MCMANUS: I think that sulfonamide sensitivity testing should be routinely performed on burn ward isolates. I also think this is not a general practice. The reason for this is perhaps that it is commonly thought that sulfonamides are not used therapeutically for anything but topical application. But, as I pointed out, with this example of plasmid-mediated resistance, I think we would all be surprised at just what kinds of multiple resistances our sulfonamide resistant bacterial population includes.

As far as sulfadiazine sensitivity of flora at the US Army Institute of Surgical Research, as I noted previously, greater than 80% of our isolates from patients who were hospitalized longer than a week were resistant to sulfadiazine. The majority of *Pseudomonas* blood isolates, for example, are sulfadiazine resistant, but I cannot say that they are treatment failures.



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